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GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

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The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. Attention was concentrated on the following areas of research during the past year: (1) Genetic exchange systems for B. anthracis; (2) The influence of the B. anthracis

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plasmid, pBA1, on the physiology and genetics of the organism; and (3) Mutant isolation and chromosomal mapping.

We have been successful in developing a mating system in B. anthracis by introducing one or more "fertility" plasmids from B. thuringiensis into the Weybridge strain. The B. anthracis transcipients that received appropriate plasmids from B. thuringiensis were, in turn, able to transfer plasmids to B. cereus or to other cells of B. anthracis. B. anthracis transcipients that acquired a plasmid encoding synthesis of the B. thuringiensis parasporal crystal produced crystals that were apparently identical to those produced by B. thuringiensis. Thus far we have not been able to demonstrate reproducibly the transfer of the B. anthracis plasmid, pBA1, by means of the mating system. However, it seems reasonably certain that further exploration of mating systems will lead to the development of strains that can transfer pBA1. The ability to transfer pBA1 from cell to cell will make it possible to study genetic manipulations of the plasmid which may lead to qualitative and/or quantitative improvements in immunizing antigen.

In addition to the loss of ability to produce protective antigen, a number of other phenotypic changes are associated with the loss of plasmid pBA1 by B. anthracis cells. It now seems probable that the change in regulation of sporulation is a primary altered characteristic and changes in colonial morphology, sensitivity to certain bacteriophages, and growth characteristics in synthetic media are direct or indirect consequences of the change in sporulation.

Our collection of auxotrophic mutants of the Weybridge strain of B. anthracis, all of which have been isolated in our laboratory, now includes those requiring indole, tryptophan, valine, leucine, phenylalanine, histidine, arginine, purines (two or three cistrons represented), pyrimidines (at least two cistrons represented), nicotinic acid, biotin, and riboflavin. By cotransduction with phage CP-51 we have found the following five linkage groups in the Weybridge strain: phe and nic, ura and pyrA, leu and his, ind and trp, and his and pur.

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SUMMARY

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The toxin-producing, but avirulent, Weybridge (Sterne) strain of B. anthracis is being used in all of our studies. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. During the past year our major effort was placed on the development of a mating system in B. anthracis. We also continued our studies on (1) the influence of plasmid pBA1 on the physiology of B. anthracis and (2) isolation of mutants and chromosomal mapping.

We have been successful in developing a mating system in B. anthracis by introducing one or more "fertility" plasmids from B. thuringiensis into the Weybridge strain. We took advantage of our transduction system using phage CP-51 to transfer the tetracycline resistance plasmid, pBC16, from B. cereus to B. thuringiensis. This allowed us to study the transfer of a plasmid that confers a selectable phenotype on its host. By making use of this plasmid and our stock of auxotrophic mutants of the Weybridge strain, we were able to identify transcipients and distinguish them from donors and recipients used in the crosses.

The Weybridge strain itself, i.e., cells that had not acquired any plasmids from B. thuringiensis, was unable to mobilize and transfer pBC16. It was able, however, to serve as a recipient in matings with cells of certain strains of B. thuringiensis. The resulting B. anthracis transcipients, which were selected on the basis of their tetracycline resistance, were found to harbor, in addition to pBC16, one or more other plasmids derived from the B. thuringiensis donor. The B. anthracis transcipients that received certain plasmids were, in turn, able to serve as donors of plasmids in matings with B. cereus or other B. anthracis cells.

B. anthracis transcipients that acquired a plasmid encoding synthesis of the B. thuringiensis parasporal crystal toxin produced crystals that were apparently identical to those produced by B. thuringiensis. The crystal-forming

B. anthracis transcipients were biologically active when compared with B. thuringiensis subsp. thuringiensis in tests against the spruce budworm. In tests for protective antigen production, crystal-positive transcipients of B. anthracis(pBA1) could not be distinguished from their parent Weybridge strain.

Thus far we have not been able to demonstrate reproducibly the transfer of pBA1 (or chromosomal DNA) by means of the mating process. However, there are many other possibilities to be explored, and as we learn more about the mechanism of the mating process, plasmid compatibilities, and plasmid functions, it seems reasonable to be optimistic that we will be able to develop an efficient system for pBA1 transfer. Among the possibilities we are considering for further study is a mating system based on the Streptococcus faecalis plasmid, $pAM_{\beta}1$.

We have spent considerable effort in attempts to find the basic physiological and genetic differences that could account for the phenotypic changes that accompany the loss of plasmid pBA1 from cells of the Weybridge strain. In addition to the loss of ability to produce protective antigen, all cured strains we have tested are similar with respect to the following characteristics: (1) Cured cells sporulate earlier and at a higher frequency than uncured cells; (2) Colonies of cured cells differ from colonies of uncured cells in their morphologyi; (3) Cured cells are more sensitive than uncured cells to certain bacteriophages; (4) Cured cells do not grow as well as uncured cells in certain synthetic media; and (5) Spore-negative mutants are found at a much higher frequency in cultures of cured strains than in cultues of uncured strains.

It now seems possible that increased frequency of sporulation may be the primary altered characteristic among those listed, and the others may be direct or indirect consequences of this change. Altered colonial morphology of cured strains is undcubtedly a direct consequence of increased sporulation. It seems likely that the increased sensitivity of cured cells to certain bacteriophages may also reflect changes in regulation of sporulation. Our original observation was that phage CP-51 plaqued with high efficiency on cells of the Weybridge strain that were cured of pBA1, but it did not form visible plaques on uncured cells. We have now learned that CP-51 will plaque on spore-negative mutants derived from uncured strains as well as from cured strains. Another significant observation is that the addition to phage assay medium of substances which inhibit sporulation, e.g. glycerol, allows plaque formation on uncured cells.

Poor growth of cured strains on certain minimal media may also reflect altered regulation of sporulation. Spore-negative mutants derived from cured strains grow much better on minimal media than their parent strains. When such mutants are transduced back to spore-positive, they resemble the parental cured type with respect to poor growth on minimal media.

It seems doubtful that cells cured of pBA1 would mutate to spore-negative more frequently than uncured cells. It seems more probable that the spore-negative mutants have a selective advantage over the spore-positive parent; i.e., they can continue to grow after the spore-positive cells are committed to sporulation.

Our collection of auxotrophic mutants derived from the Weybridge strain now includes mutants with lesions in genes required for the synthesis of indole, tryptophan, valine, leucine, phenylalanine, histidine, arginine, purines, pyrimidines, nicotinic acid, biotin, and riboflavin. Thus far we have established five linkage groups by cotransduction with phage CP-51. These include mutations in genes required for synthesis of (1) phenylalanine and nicotinic acid, (2) uracil and carbamyl phosphate, (3) leucine and histidine, (4) indole and tryptophan, and (5) histidine and purine.

Foreword

Citation of trade names in this report does not constitute an official Department of the Army endorsement or approval of the use of such items.

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This is the fourth annual report submitted under contract No. DAMD 17-80-C-0099. Research on the contract began July 1, 1980. The contract was extended for a second year beginning July 1, 1981 and on July 1, 1982 it was extended a second time for a period of two years. The previous annual reports are dated December 1980, December 1981, and December 1982.

During the year represented by this annual report our research concentrated largely on the development of a mating system for the transfer of plasmids by B. anthracis. Other areas of investigation were concerned with (1) the influence of the B. anthracis plasmid, pBA1, on the physiology of the organism and (2) isolation of mutants and chromosomal mapping by transduction. In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

MATERIALS AND METHODS

Organisms. The Weybridge (1) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England in 1957. It was isolated by Sterne (2) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Table 1 lists specific strains and mutants referred to in this report.

Media. For convenience to the reader compositions of the various culture media referred to in this report are given in detail below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

PA (phage assay) broth: Nutrient broth (Difco), 8 g; NaCl, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.15 g. The pH was adjusted to 6.0 with HCl.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g. The pH was adjusted to 7.0 with NaOH.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 6 g; K_2HPO_4, 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g; $MgSO_4.7H_2O$, 0.2 g; $FeCl_3.6H_2O$, 0.04 g; $MnSO_4.H_2O$, 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and $FeCl_3$ were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal M: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Antisera. All antisera were kindly supplied by Dr. Anna Johnson of USAMRIID.

Protective antigen assays. These were carried out by the agar diffusion method as outlined by Thorne and Belton (1).

Propagation and assay of phage CP-51. The methods described by Thorne (3) were followed. The indicator for routine assay of CP-51 was B. cereus NRRL B-569.

Procedures used in mating experiments. Cells for mating were grown in 25 ml of broth in a 250-ml flask incubated at 30° C. on a reciprocal shaker. After 15 hours of incubation, 0.3 ml of culture was transferred to 25 ml of fresh broth and incubation was continued for 8 to 10 hours. The cells were then diluted 1:50 in the same kind of broth used for their growth. One ml of donor and one ml of recipient were incubated together in 20-mm tubes on a shaker at 30° C. for the desired period of time and samples were plated on appropriate selective media. Variations in this procedure and the kinds of broth used in particular experiments are given in the text.

RESULTS AND DISCUSSION

I. Development of a mating system in B. anthracis

Gonzalez, Brown, and Carlton (4) reported the transfer of B. thuringiensis plasmids among strains of B. thuringiensis and B. cereus by some undefined

Table 1

Strains and mutants referred to in this report

Organism	Characteristics and/or source
Bacillus anthracis	
Weybridge	Avirulent Sterne-type strain, obtained from MRE, Porton, England
Weybridge A	Colonial variant isolated from Weybridge
Weybridge A M2	Ind^- mutant of Weybridge A
Weybridge A M2 tr244-1	Cry^+ transcipient of M2. See text.
Weybridge A M2 td1	Ind^- Tet^r $(pBC16)^+$ by transduction of M2
Weybridge A M4	Nic^- mutant of Weybridge A
Weybridge A M11	Leu^- mutant of Weybridge A
Weybridge A M14	Phe^- mutant of Weybridge A
Weybridge A M17	Ade^- mutant of Weybridge A
Weybridge A M17 tr245-4	Cry^+ transcipient of M17. See text.
Weybridge A M18	*pyrA* mutant of Weybridge A
Weybridge A M18 td1	*pyrA* Tet^r $(pBC16)^+$ by transduction
Weybridge A M18 td1 cured 28	*pyrA* Tet^r $(pBA1)^-$ from M18 td1
Weybridge A M18 td2	*pyrA* Tet^r $(pBC16)^+$ by transduction
Weybridge A M18 td2 cured 25	*pyrA* Tet^r $(pBA1)^-(pBC16)^-$ from M18 td2
Weybridge A M18 td2 cured 25 *str-1*	Str^r mutant of M18 td2 cured 25
Weybridge A M18-M9	*pyrA* Ind^- mutant of M18
Weybridge A M23	Ura^- mutant of Weybridge A
Weybridge A M23 cured 1	Ura^- $(pBA1)^-$ from M23
Weybridge A M23 cured 1 td1	Ura^- $(pBA1)^-$ Tet^r $(pBC16)^+$ by transduction
Weybridge A M23 cured 2	Ura^- $(pBA1)^-$ from M23
Weybridge A M23 tr237-3	Cry^+ transcipient of Weybridge A M23 cured 2. See text.
Weybridge A M23 tr247-10	Cry^+ transcipient of Weybridge A M23 cured 2. See text.

(Continued)

Table 1 (continued)

Weybridge A M693	His$^-$ mutant of Weybridge A
Weybridge A M714	Trp$^-$ mutant of Weybridge A
Weybridge A M715	Pur$^-$ mutant of Weybridge A
Weybridge M44	Trp$^-$ mutant of wild-type Weybridge
Weybridge M44 td1	Trp$^-$ Tet^r (pBC16)$^+$ by transduction
Weybridge M44 str-1	Str^r mutant of M44
Weybridge M44 tr84-6	Transcipient of M44 str-1. See text.
Weybridge M44 tr112-9	Transcipient of M44 str-1. See text.
Weybridge M44 tr203-23	Transcipient of M44 str-1. See text.
Weybridge M44 tr203-24	Transcipient of M44 str-1. See text.
Weybridge M44 tr203-27	Transcipient of M44 str-1. See text.
Weybridge M44 tr243-8	Transcipient of M44. See text.
Bacillus cereus	
569	U.S.D.A. Laboratory, Peoria, Illinois (NRRL)
569 M20	Anthranilic acid$^-$ mutant of 569
569 M20 str-2	Str^r mutant of 569 M20
GP7	Carries pBC16. K. Bernhard.
Bacillus thuringiensis	
4040	subsp. finitimus. NRRL
4042A	subsp. thuringiensis. NRRL
4042A M8	Ade$^-$ mutant of 4042A
4042A M8 td1	Ade$^-$ Tet^r (pBC16)$^+$ by transduction
4042A M8 td2	Ade$^-$ Tet^r (pBC16)$^+$ by transduction
4042A M8-13	Cry$^-$ oligosporogenous (Osp) mutant of M8
4042A M8-13 td1	Cry$^-$ Osp Tet^r (pBC16)$^+$ by transduction
4042A M8-13 td2	Cry$^-$ Osp Tet^r (pBC16)$^+$ by transduction
4042B	subsp. aizawai. NRRL
4042B M45	Trp$^-$ mutant of 4042B
4042B M45 td1	Trp$^-$ Tet^r (pBC16)$^+$ by transduction

(Continued)

Table 1 (continued)

4043	subsp. dendrolimus. NRRL
4043 tdl	Tet^r $(pBC16)^+$ by transduction
4043 td2	Tet^r $(pBC16)^+$ by transduction
4049	subsp. morrisoni. NRRL
4049 tdl	Tet^r $(pBC16)^+$ by transduction
4050	subsp. tolworthi. NRRL
4050 tdl	Tet^r $(pBC16)^+$ by transduction
4050 td2	Tet^r $(pBC16)^+$ by transduction
4059	subsp. toumanoffi. NRRL
4059 tdl	Tet^r $(pBC16)^+$ by transduction
13367	ATCC
13367 tdl	Tet^r $(pBC16)^+$ by transduction
33740	ATTC
33740 tdl	Tet(r) $(pBC16)^+$ by transduction
BTI	subsp. israelensis. M. deBarjac
BTI tdl	Tet(r) $(pBC16)^+$ by transduction
HD-1	subsp. kurstaki. A. Yousten.
HD-1 tdl	Tet(r) $(pBC16)^+$ by transduction
YAL	subsp. alesti. A. Yousten
YAL tdl	Tet(r) $(pBC16)^+$ by transduction
Bacillus subtilis	
BQ838	MLS^r Carries pAM_B1. O. Landman
Streptococcus anginosis	MLS^r Carries pAM_B1. O. Landman

mechanism. Transfer occurred during mixed growth of donor and recipient strains. They did not study transfer of any plasmids with selectable markers; rather they recovered recipients which were originally parasporal crystal-negative (Cry^-) and examined them by phase microscopy for the presence of toxin crystals. Cry^+ "transcipients" were then analyzed for the presence of new plasmids which were not present in recipients before mating.

We have taken advantage of the B. thuringiensis mating system described by Gonzalez, et al.(4) to develop a similar type of mating system in B. anthracis. We have found that certain plasmids which apparently are responsible for the mating event in B. thuringiensis are also effective in B. anthracis, i.e., B. anthracis transcipients derived from crossing B. thuringiensis with B. anthracis are, in turn, effective donors. Our approach was a little different from that of Gonzalez, et al. in that we examined the mobility of the tetracycline resistance plasmid, pBC16. We moved pBC16 into potential donors by transduction with CP-51. By using donors that carried pBC16 and recipients which were resistant to streptomycin by virtue of a chromosomal mutation, we were able to select for transcipients by plating mating mixtures on agar containing both streptomycin and tetracycline.

Initial survey of B. thuringiensis strains for effective donors. In our first experiments we tested a number of B. thuringiensis strains for the ability to transfer pBC16 to B. cereus 569 M20 str-2. The idea was to identify a donor that was efficient in transferring plasmids to B. cereus and then test it for the ability to transfer to B. anthracis.

The results of this initial survey are shown in Table 2. It should be pointed out that nutrient broth was used in these tests for growing the donors and recipients. (In experiments reported below we found that nutrient broth was not the best medium for growing cells to be used in matings). In the results shown in Table 2 B. thuringiensis 4042A M8-13 tdl (pBC16) and subsp. israelensis tdl (pBC16) were by far the best donors among the several strains tested. Several representative transcipients from mating mixtures in which 4042A M8-13 tdl or subsp. israelensis tdl was the donor were tested for auxotrophic markers and for the presence of pBC16. All transcipients examined required anthranilic acid for growth, showing that they originated from B. cereus 569 M20, and in addition, they all carried pBC16 as determined by electrophoresis of DNA extracts in agarose gels. Table 2 shows also that B. anthracis mutants carrying pBC16 were ineffective in transferring the plasmid to B. cereus. This confirms

Table 2

Results of testing various strains of B. thuringiensis and B. anthracis as donors of pBC16 to B. cereus 569 M20 str-2

Strains tested	Tet^r colonies per ml[1]
B. thuringiensis	
4042B M45 tdl(pBC16)	300
4042A M8 tdl(pBC16)	0
4042A M8-13 tdl(pBC16)	4×10^4
HD-1 tdl(pBC16)	70
4043 tdl(pBC16)	0
4049 tdl(pBC16)	100
BTI tdl(pBC16)	5×10^4
33740 tdl(pBC16)	500
YAL tdl(pBC16)	200
4050 tdl(pBC16)	0
B. anthracis	
Weybridge A M18 tdl(pBC16)	0
Weybridge A M23 tdl(pBC16)	0
Weybridge A M23$(pBA1)^-$ tdl(pBC16)	0
Weybridge M44 tdl(pBC16)	0

[1]Samples were plated on L agar containing streptomycin and tetracycline at 21 and 48 hours. The numbers of transcipients were similar at the two sampling times and the numbers given represent average values. Control samples of donors and recipient incubated alone yielded no colonies on the selective medium.

that neither pBC16 nor the B. anthracis plasmid, pBA1, is capable of inducing the mating event.

Transfer of plasmids from B. thuringiensis to B. anthracis. Once we found that 4042A M8-13 and subsp. israelensis were effective donors in matings with B. cereus, we then tested them for the ability to transfer pBC16 to B. anthracis. Some of these results are shown in Table 3. Both strains were effective in transferring pBC16 to the two mutants of the Weybridge A strain that were tested. In all of a large number of transcipients tested the auxotrophic marker of the respective recipient was retained and the presence of pBC16 was confirmed by gel electrophoresis. In addition to pBC16, the transcipients carried one or more other plasmids derived from the donor. In some transcipients as many as five plasmid bands were found in addition to pBC16 and pBA1.

Effect of growth medium on frequency of pBC16 transfer. Comparisons of pBC16 transfer from B. thuringiensis to B. cereus and B. anthracis with cells grown in three different media are shown in Table 4. In crosses betwen B. thuringiensis and B. anthracis the numbers of tetracycline-resistant (Tet^r) transcipients obtained with cells grown in L-broth or (BHI) broth were dramatically greater than the numbers obtained with cells grown in nutrient broth. The effect of growth medium was not as dramatic in matings between B. thuringiensis and B. cereus; nutrient broth was better than L-broth, but BHI broth was better than nutrient broth. As a consequence of these results we chose BHI broth for growth of recipient and donor cells for routine mating experiments.

Transfer of pBC16 by B. anthracis transcipients. Table 5 shows that B. anthracis transcipients were effective donors of pBC16 to B. cereus and to other cells of B. anthracis. In our system of labeling transcipients for identification, e.g. Weybridge M44 tr 84-6, Weybridge M44 designates the recipient from which the transcipient was derived, tr stands for transcipient, 84 is the mating mixture number, and 6 is the number of the particular transcipient that has been purified by single colony isolation. The table includes data comparing the frequencies of pBC16 transfer by B. anthracis transcipients derived from two different subspecies of B. thuringiensis. B. thuringiensis subsp. israelensis carries seven or eight plasmids, and strain 4042A, subsp. thuringiensis, carries five or six. All transcipients thus far examined have been found to contain one or more plasmids derived from B. thuringiensis. We have not yet determined which plasmids are responsible for the transfer process. One plasmid of

Table 3

Transfer of pBC16 from B. thuringiensis to B. anthracis

Donor	Recipient	Tet^r Str^r colonies per ml*
B. thuringiensis 4042A M8-13 tdl(pBC16)	*B. cereus* 569 M20 *str-2*	2.7×10^4
" "	Weybridge M44 *str-1*	2.3×10^3
" "	Weybridge A M18 td2 cured $25(pBA1)^-(pBC16)^-$ *str-1*	1.0×10^3
B. thuringiensis BTI tdl(pBC16)	*B. cereus* 569 M20 *str-2*	2.0×10^4
" "	Weybridge M44 *str-1*	3.3×10^2
" "	Weybridge A M18 td2 cured $25(pBA1)^-(pBC16)^-$ *str-1*	2.5×10^2

*Mating mixtures were plated after 21 hours on L agar plus streptomycin and tetracycline. Donor and recipient cultures incubated alone yielded no colonies on the selective medium.

Table 4

Effect of growth medium on numbers of transcipients in mating mixtures*

Recipient	Medium	Tet^r transcipients per ml
B. cereus 569 M20 str-2	Nutrient broth	1.2×10^5
	L-broth	3.5×10^4
	BHI broth	2.7×10^5
B. anthracis Weybridge A M2	Nutrient broth	3.7×10^2
	L-broth	2.4×10^4
	BHI broth	1.0×10^4
B. anthracis Weybridge A M4	Nutrient broth	6.6×10^2
	L-broth	3.5×10^3
	BHI broth	1.3×10^4

*The donor was *B. thuringiensis* 4042A M8-13 tdl(pBC16). Cells were grown for 9 hours following transfer from 15-hour cultures. For preparing mating mixtures cells were diluted 1:50 in the same kind of medium in which they were grown. Selective media were: L-agar plus 200 ug of streptomycin and 25 ug of tetracycline per ml for 569 M20; Min 1C plus 40 ug of tryptophan, 10 ug of nicotinic acid, and 10 ug of tetracycline per ml for Weybridge A M2 and M4. The numbers of transcipients represent averages of those found in samples plated at 21 hours and 39 hours.

Table 5

Transfer of pBC16 from B. anthracis transcipients derived from two subspecies of B. thuringiensis: Comparison of transfer frequencies

Donor*	Recipient	Tet^r transcipients per ml
Weybridge M44 tr84-6	*B. cereus* 569 M20 Ant^- *str-2*	5.7×10^3
"	Weybridge A M23 Ura^- $(pBC16)^-$	4.1×10^2
"	Weybridge A M18 *pyrA* $(pBC16)^-$	6.1×10^2
"	Weybridge A M14 Phe^- $(pBC16)^-$	2.0×10^2
Weybridge M44 tr112-9	*B. cereus* 569 M20 Ant^- *str-2*	2.4×10^5
"	Weybridge A M23 Ura^- $(pBC16)^-$	4.5×10^4
"	Weybridge A M18 *pyrA* $(pBC16)^-$	5.7×10^4
"	Weybridge A M14 Phe^- $(pBC16)^-$	7.2×10^4

*Weybridge M44 tr84-6 is a transcipient isolated from a mating mixture in which *B. thuringiensis* subsp. *thuringiensis* strain 4042A M8-13 tdl(pBC16) was the donor and Weybridge M44 *str-1* was the recipient. Weybridge M44 tr112-9 is a transcipient isolated from a similar mating mixture in which the donor was *B. thuringiensis* subsp. *israelensis* strain BTI tdl(pBC16).

subspecies israelensis which seems to be a likely candidate is probably slightly larger than pBAl, based on distances of electrophoretic migration in agarose gels. Two plasmids from strain 4042A which are likely candidates are both smaller than pBAl.

Transcipient M44 tr 84-6 in Table 5 was derived from a mating mixture of Weybridge M44 str-1 and B. thuringiensis 4042A M8-13(pBC16) subsp. thuringiensis. Transcipient M44 tr 112-9 was derived from a similar mating mixture in which B. thuringiensis subsp. israelensis(pBC16) was the donor donor. As Table 5 shows, fewer Tet^r transcipients were isolated from mixtures having M44 tr 84-6 as the donor than from those having M44 tr 112-9 as the donor. The data also show that B. cereus was a more effective recipient than B. anthracis regardless of which donor was used. This was true in all experiments in which the two strains were compared as recipients.

Time and frequency of pBC16 transfer in matings between B. thuringiensis and B. cereus. Until recently we had always followed the procedure of Gonzalez, et al.(4) and sampled mating mixtures to select transcipients after approximately 20 to 24 hours of incubation and again after approximately 44 hours. We have now found that in mating mixtures of B. thuringiensis with B. cereus, transcipients are formed much earlier than we had expected. Some results showing the numbers of transcipients found after various times of incubation are shown in Table 6. In the experiments shown there the donor was B. thuringiensis 4042A M8-13(pBC16), and the recipient was B. cereus 569 M20 str-2. Cultures grown in BHI broth for 8 to 9 hours were diluted 1:50 and 1 ml of donor and 1 ml of recipient were mixed and incubated at 30°C. Control tubes contained 1 ml of donor or recipient and 1 ml of BHI broth; these were used for viable cell determinations and testing for spontaneous acquisition of tetracycline resistance by the recipients and spontaneous acquisition of streptomycin resistance by the donors. Spontaneous Tet^r or Str^r mutants of these strains were found only rarely. Transcipients were selected on (1) L-agar containing 200 ug of streptomycin and 25 ug of tetracycline per ml and (2) Min IC agar containing 40 ug of anthranilic acid and 25 ug of tetracycline per ml. Numbers scored on the two media were not significantly different; averages are given in the table. Frequencies of transcipients were calculated by dividing the number of transcipients by the number of recipients. A few transcipients (about 100 per ml) were found at two hours which was the earliest time tested. During the period between two and four hours the number increased to about 3×10^5, which

Table 6

Time and frequency of pBC16 transfer in matings between B. thuringiensis and B. cereus*

Experiment No.	Incubation time (hours)	Donors per ml	Recipients per ml	Tet^r transcipients	
				No. per ml	Frequency
1	2			1.2×10^2	
	4			2.0×10^5	
	6			3.3×10^5	
	8			5.5×10^5	
	10			5.9×10^5	
2	0	5.8×10^6	6.0×10^6		
	2	2.5×10^7	3.2×10^7	8.5×10^1	2.7×10^{-6}
	4	5.5×10^7	5.8×10^7	2.5×10^5	4.3×10^{-3}
	6	5.5×10^7	5.5×10^7	5.2×10^5	9.5×10^{-3}
	15	8.0×10^8	4.0×10^8	6.0×10^5	1.5×10^{-3}
3	0	1.9×10^7	2.2×10^7		
	2	6.2×10^7	7.4×10^7	1.7×10^2	2.3×10^{-6}
	4	2.4×10^8	1.1×10^8	3.9×10^5	3.5×10^{-3}
	6	4.4×10^8	1.9×10^8	1.2×10^6	6.3×10^{-3}
	15	6.0×10^8	9.0×10^8	3.9×10^6	4.3×10^{-3}

*The donor was *B. thuringiensis* 4042A M8-13 td1(pBC16) and the recipient was *B. cereus* 569 M20 *str-2*.

represented a frequency of 4×10^{-3} based on recipient cell count. After four hours the numbers of transcipients increased only gradually with time. The highest frequency occurred at six hours. Beyond that time the number of transcipients increased only slightly; the frequency actually decreased as a result of increase in number of recipients. It is important to point out that all of a large number of transcipients tested retained the anthranilic acid requirement of the recipients.

The fact that the number of transcipients increased by more than three logs during the period between two and four hours means that many independent transfer events occurred, and thus frequency determinations at the early hours are meaningful. This information will be useful in studies on the mechanism of the transfer process. For mechanism studies it will now be possible to avoid long incubation periods which tend to make it difficult to interpret results. We have not yet had time to test mating mixtures containing B. anthracis as donor and/or recipient for time of transfer. This will be done in the near future.

A second survey of B. thuringiensis strains for effective donors. In the results reported above the frequencies of transfer of the tetracycline resistance plasmid, pBC16, were higher with subsp. israelensis as donor than with the mutant of subsp. thuringiensis as donor. B. anthracis transcipients derived from either donor were in turn effective donors of pBC16. However, in neither system were we able to demonstrate transfer of the B. anthracis plasmid, pBA1, to either B. cereus or strains of B. anthracis cured of the plasmid. We therefore decided to look again at the other strains of B. thuringiensis in my collection with the hope of finding a system in which pBA1 would be transferred efficiently.

In our first test of a number of strains for their ability to serve as donors, we used cells grown in nutrient broth. However, as reported above, we later learned that BHI broth was a much more effective growth medium for cells to be mated. Therefore, in the second survey of our strains we used BHI broth for growing donor and recipient cells. The strains that were to be tested as donors were first infected with the tetracycline resistance plasmid, pBC16, by transduction with phage CP-51. The recipients were resistant to streptomycin. Transcipients were selected after 22 hours by plating mating mixtures on L agar containing 200 ug of streptomycin and 5 or 10 ug of tetracycline per ml.

The results of these tests are shown in Table 7. Some strains that

Table 7

Test of various strains of B. thuringiensis as donors of pBC16 in mating mixtures with B. cereus and B. anthracis

Donor strain of *B. thuringiensis*	Tet^r transcipients per ml when mated with[a]	
	B. cereus 569 M20 str-2	*B. anthracis* Weybridge M44 str-1
ATCC 33740 tdl(pBC16)	80	0
YAL tdl(pBC16) subsp. *alesti*	4×10^5	60
HD-1 tdl(pBC16) subsp. *kurstaki*	100	0
4059 tdl(pBC16) subsp. *toumanoffi*	6.4×10^3	1.1×10^4
4042A M8 td2(pBC16) subsp. *thuringiensis*	1.5×10^4	3.2×10^4
4042A M8-13 tdl(pBC16) subsp. *thuringiensis*	1.8×10^6	1.7×10^4
4050 td2(pBC16) subsp. *tolworthi*	15	0
4049 tdl(pBC16) subsp. *morrisoni*	3.0×10^4	1.7×10^4
4043 td2(pBC16) subsp. *dendrolimus*	0	0
4042B M45 tdl(pBC16) subsp. *aizawai*	100	0
ATCC 13367 tdl(pBC16)	0	0

[a] Transcipients were selected on L agar containing 200 ug of streptomycin and 10 ug of tetracycline per ml. Control tubes in which each strain was incubated with 1 ml of BHI broth yielded no spontaneous Tet^r Str^r colonies.

demonstrated little or no donor activity in the previous test were much more effective in the more recent test. Only two of the 11 strains tested failed to show donor activity. There appeared to be some specificity among the various strains. For example, subsp. alesti was very active as a donor to B. cereus, but it was barely active with B. anthracis. Although 4042A M8-13 was reasonably effective with B. anthracis, it gave 100 times more transcipients with B. cereus. Strains 4049, 4059, and 4042A M8 gave similar numbers of transcipients with both recipient strains. It was observed that some transcipients of B. cereus and B. anthracis derived from matings in which 4042A M8(pBC16) was the donor formed parasporal crystals. This donor was therefore the first chosen for further investigation. It should be emphasized that the presence of the specific auxotrophic markers in the transcipients permitted their positive identification. The presence of pBC16 was confirmed in a number of transcipients by electrophoresis of lysates in agarose gels. Although tetracycline-resistant mutants of B. cereus 569 occur spontaneously at a low frequency, we have never observed such spontaneous mutants of the Weybridge strain of B. anthracis.

Transfer of plasmids by primary crystal-positive B. anthracis transcipients. Transcipients isolated from mating mixtures in which B. thuringiensis was the donor are referred to as primary transcipients. Secondary transcipients are those derived from matings in which the donors were B. cereus or B. anthracis transcipients carrying one or more "fertility" plasmids originally transferred from B. thuringiensis.

Table 8 shows the donor activity of some primary Cry^+ transcipients derived from mating mixtures with B. thuringiensis 4042A M8(pBC16) as donor and B. anthracis Weybridge M4 str-1 as recipient. The three independently isolated transcipients tested gave similar results. Between 10^4 and 10^5 Tet^r transcipients per ml were isolated from matings in which Weybridge A M23(pBA1)$^-$ was the recipient. This represents a frequency of 0.1% to 0.5% based on the number of recipients. With B. cereus 569 str-2 as the recipient more than 10^7 transcipients per ml were produced, representing frequencies of 5% to 8% based on the number of recipients. The retention of specific auxotrophic markers in the transcipients distinguished them from donors and the presence of pBC16 distinguished them from recipients.

Transfer of plasmids by secondary crystal-positive B. anthracis transcipients. Table 9 shows some results of mating secondary Cry^+ B. anthracis

Table 8

Effectiveness of primary crystal-positive B. anthracis transcipients as donors in the transfer of plasmids

Mating mixture No.	Donor	Recipient	Tet^r transcipients per ml[a]
231	Weybridge M44 tr 203-23 Cry$^+$ Ind$^-$ str-1 (pBA1)(pBC16)	Weybridge A M23 (pBA1) Ura$^-$	2.8×10^4
233	" "	B. cereus 569 str-2 Ant$^-$	2.6×10^7
235	Weybridge M44 tr 203-24 Cry$^+$ Ind$^-$ str-1 (pBA1)(pBC16)	Weybridge A M23 (pBA1) Ura$^-$	3.1×10^4
236	" "	B. cereus 569 str-2 Ant$^-$	2.0×10^7
237	Weybridge M44 tr 203-27 Cry$^+$ Ind$^-$ str-1 (pBA1)(pBC16)	Weybridge A M23 (pBA1) Ura$^-$	1.4×10^5
238	" "	B. cereus 569 str-2 Ant$^-$	4.0×10^7

[a]Mating mixtures were plated after 24 hours on hydrolyzed casein agar containing 5 µg of tetracycline per ml and supplemented with uracil or anthranilic acid to allow growth of transcipients. The donor cells were Ind$^-$ and thus could not grow on the selective agar. Control tubes containing donor or recipient cells alone yielded no colonies on the selective media.

Table 9

Effectiveness of secondary crystal-positive B. anthracis transcipients as donors in the transfer of plasmids

Mating mixture No.	Donor	Recipient	Tet^r transcipients per ml	Frequency[a]
240	Weybridge A M23 tr237-3 $(pBA1)^-$ Ura^- Cry^+	Weybridge M44 (pBA1) Ind^-	5.1×10^6	1.1×10^{-1}
241	" "	Weybridge A M2 (pBA1) Ind^-	5.4×10^4	1.2×10^{-3}
242	" "	Weybridge A M17 (pBA1) Ade^-	3.6×10^4	8.0×10^{-4}
243	Weybridge A M23 tr237-10 $(pBA1)^-$ Ura^- Cry^+	Weybridge M44 (pBA1) Ind^-	6.2×10^6	1.4×10^{-1}
244	" "	Weybridge A M2 (pBA1) Ind^-	5.0×10^4	1.1×10^{-3}
245	" "	Weybridge A M17 (pBA1) Ade^-	3.4×10^4	7.5×10^{-4}
246	Weybridge M44 tr243-8 (pBA1) Ind^- Cry^+	Weybridge A M23 $(pBA1)^-$ Ura^- *str-1*	4.7×10^4	1.0×10^{-3}
247	" "	*B. cereus* 569 M20 Ant^- *str-2*	1.3×10^7	2.6×10^{-2}

Table 9 (Continued)

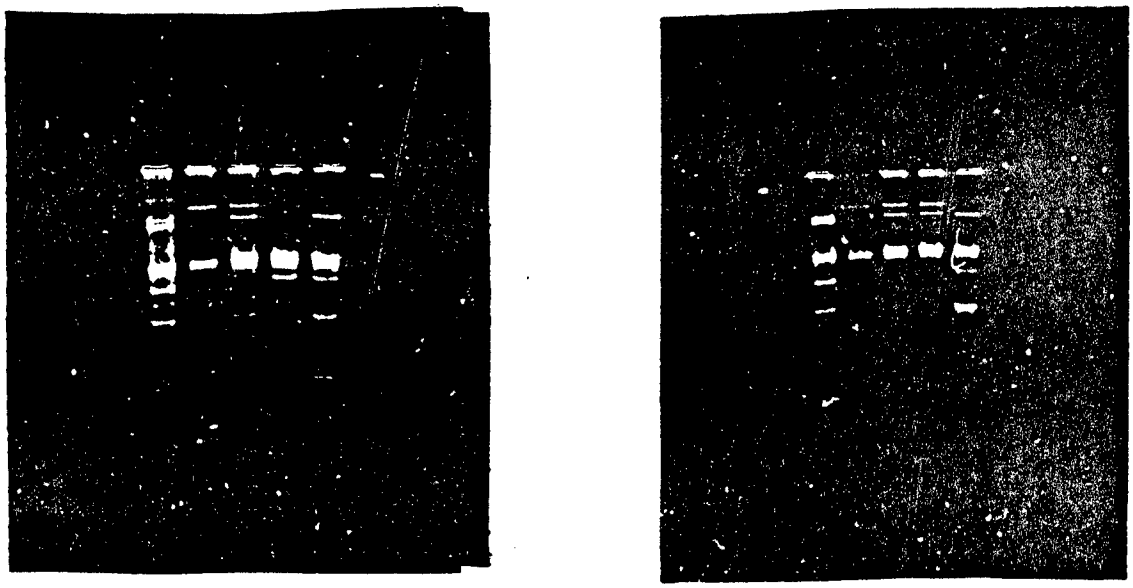
248	Weybridge A M2 tr244-1 (pBA1) Ind$^-$ Cry$^+$	Weybridge A M23 (pBA1)$^-$ Ura$^-$ *str-1*	4.5×10^4	1.0×10^{-3}
249	" "	*B. cereus* 569 M20 Ant$^-$ *str-1*	4.3×10^7	8.6×10^{-2}
250	Weybridge A M17 tr245-4 (pBA1) Ade$^-$ Cry$^+$	Weybridge A M23 (pBA1)$^-$ Ura$^-$ *str-1*	5.6×10^4	1.2×10^{-3}
251	" "	*B. cereus* 569 M20 Ant$^-$ *str-2*	5.5×10^7	1.1×10^{-1}

[a]Frequencies are calculated by dividing the number of transcipients per ml by the number of recipients per ml. The average number of *B. cereus* recipients was 5.0×10^8 CFU per ml and that of *B. anthracis* recipients was 4.5×10^7 CFU per ml. *B. anthracis* grows in much longer chains than *B. cereus*.

transcipients with B. cereus and B. anthracis. The data show that secondary transcipients were as effective as primary transcipients in transferring pBC16. A conclusion to be drawn from Table 9 which may be very significant is that Weybridge M44 was a much better recipient than the Weybridge A mutants, M2 and M17. The frequencies of transfer obtained with Weybridge M44 as recipient were 11% and 14%, which are similar to the frequencies obtained with B. cereus 569 as recipient, i.e., 3% to 11%. In previous experiments with donors derived from B. thuringiensis subsp. israelensis or B. thuringiensis 4042A M8-13, B. cereus was always a better recipient than B. anthracis. Although Table 9 shows that the actual number of transcipients was higher for B. cereus 569 than for B. anthracis M44, the frequencies, based on numbers of recipients as colony-forming units, were similar for the two recipients. The number of colony-forming units was always higher for B. cereus than for B. anthracis. Undoubtedly this reflects the fact that B. anthracis grows in much longer chains than B. cereus 569. Nevertheless, these frequencies are the highest we have obtained for B. anthracis and they are very encouraging for future work.

Plasmid profiles of donors, recipients, and transcipients. Plasmids are routinely detected by electrophoresis of extracts in 0.7% agarose gels and staining with ethidium bromide. Photographs of some gels showing the plasmid profiles of various donors, recipients, and transcipients are reproduced in Figure 1, 2, and 3. Figure 1A shows plasmid profiles of the donor, B. thuringiensis 4042A M8-13(pBC16); the recipients, B. anthracis Weybridge A M2(pBA1) and B. cereus 569 M20 str-2; and transcipients derived from each of the recipients. Chromosomal DNA can be recognized as the most prominent and relatively diffuse band in each lane. The two bands at the lower end of lanes 1, 3, and 5 are two forms of pBC16, the tetracycline resistance plasmid, which provided the basis for selecting transcipients. B. thuringiensis 4042A M8-13(pBC16) in lane 1 has, in addition to pBC16, at least 6 plasmid bands. Weybridge A M2, shown in lane 2, has only one band, pBA1, which migrated a bit farther than the top band of 4042A M8-13. The Weybridge A M2 transcipient in lane 3 has, in addition to pBC16, at least four other bands corresponding to ones found in M8-13. B. cereus 569 M20 str-2 in lane 4 has only one plasmid which migrated slightly faster than chromosomal DNA. The B. cereus transcipient in lane 5 has, in addition to pBC16, at least two other bands corresponding to ones in M8-13 and in the B. anthracis transcipient.

The gel in Figure 1B is similar to that in Figure 1A except that Weybridge



A B

Figure 1. Agarose gel electrophoresis of plasmid extracts

A. Lane 1: B. thuringiensis 4042A M8-13(pBC16)
Lane 2: B. anthracis Weybridge AM2(pBA1)
Lane 3: Weybridge AM2 transcipient, tr 135-1, from the cross: M8-13(pBC16) x AM2
Lane 4: B. cereus 569 M20 str-2
Lane 5: B. cereus 569 transcipient from the cross: M8-13(pBC16) x 569 M20 str-2

B. Lane 1: B. thuringiensis 4042A M8-13(pBC16)
Lane 2: B. anthracis Weybridge M44 str-1
Lane 3: Weybridge M44 transcipient, tr 84-6, from the cross: M8-13(pBC16) x Weybridge M44
Lane 4: Weybridge M44 transcipient, tr 84-7, from the above cross
Lane 5: B. cereus 569 M20 str-2 transcipient, tr 152-22, from the cross: Weybridge M44 tr 84-6 x 569 M20 str-2

M44 is in lane 2; two M44 transcipients from a cross in which M8-13(pBC16) was the donor are in lanes 3 and 4; and the B. cereus transcipient in lane 5 was from a mating in which the B. anthracis transcipient shown in lane 3 was the donor. The respective plasmid profiles are similar to those in Figure 1A.

In agarose gels B. thuringiensis 4042A M8-13(pBC16) routinely shows three characteristic bands above the chromosomal DNA band. [In the gel pictures in Figure 1A and 1B the second and third bands found in M8-13 (lane 1 in each gel) are very close together. This is the usual result when electrophoresis is carried out at 50 volts. However, the two corresponding bands are separated much better in the gel shown in Figure 2 which was run at 70 volts]. The slower moving of these two bands corresponds to the band below pBA1 in the B. anthracis transcipients and the only band above the chromosomal DNA in the B. cereus transcipient. For purposes of reference we have tentatively designated this plasmid as pBT1. We have analyzed a considerable number of B. cereus and B. anthracis transcipients derived directly from M8-13(pBC16) as donor or from primary or secondary transcipients as donors and this plasmid has been found in a large majority of them. In contrast, we have never observed the band which is directly below pBT1 in M8-13(pBC16) to be present in transcipients. The smaller B. thuringiensis plasmids which migrate faster than chromosomal DNA are found in transcipients and their distribution appears to be more or less random. Because pBT1 is very frequently found in transcipients derived from M8-13(pBC16) as donor, it is tempting to speculate that it is a "fertility" plasmid responsible for the donor characteristics of M8-13(pBC16). Although this has not been tested, we are in a position to do so by comparing donor abilities of transcipients displaying various plasmid profiles. We plan to do such studies in the future in connection with our efforts to learn the mechanism of the mating process.

B. thuringiensis 4042A wild type and 4042A M8, an Ade^- mutant isolated from 4042A following mutagenesis with UV light, produce the typical B. thuringiensis parasporal crystal which is toxic to certain insects. M8-13 is an oligosporogenous (Osp) and acrystalliferous mutant isolated from M8. It is converted by phage TP-13 to spore-positive, crystal-positive at a high frequency (5). The lesion in M8-13 which renders it Osp Cry^- is very likely a single chromosomal mutation, and we have considerable evidence that the mutation is in an RNA polymerase gene (Perlak, F. J. and C. B. Thorne, unpublished). As with all the strains we have tested as donors in mating mixtures with B. cereus or B.

anthracis, we introduced the selectable tetracycline resistance plasmid, pBC16, into M8-13 by transduction with phage CP-51. We expected to find crystals in some of the B. cereus and B. anthracis transcipients derived from mating mixtures in which M8-13(pBC16) was the donor; however, in our examination of a large number of such transcipients by phase microscopy we failed to find any crystals.

In contrast, when we began to use 4042A M8(pBC16) as a donor we observed crystals in transcipients without any difficulty. The reason for this apparent discrepancy came to light when we carefully examined plasmid profiles of 4042A and its derivatives. Figure 2 is a reproduction of a photograph of an agarose gel in which plasmid profiles of the organisms in question were examined. The gel shows that a plasmid which is present in 4042A, 4042A M8, and 4042A M8-13 (lanes 1, 2, and 3) is absent in the two tetracycline-resistant transductants, M8-13 td1(pBC16) and M8-13 td2(pBC16) shown in lanes 4 and 5. It is present, however, in tetracycline-resistant transductants of 4042A M8, i.e. 4042A M8 td1(pBC16) and 4042A M8 td2(pBC16) shown in lanes 6 and 7. It is also present in the B. cereus transcipient (lane 8) derived from a mating mixture in which 4042A M8 td2(pBC16) was the donor.

The gel pictures in Figure 3A and 3B confirm the correlation between the presence of this plasmid, which we will refer to as pBT2, and the ability of cells to produce parasporal crystals. In the agarose gels pBT2 forms a band directly below pBA1 (lanes 1-7 in Figure 3A) and above pBT1 (lane 8). Among all transcipients thus far examined we have found pBT2 to be present in those that are Cry^+ and absent in those that are Cry^-. The frequency of Cry^+ colonies was very low among transcipients isolated from mating mixtures in which B. thuringiensis 4042A M8(pBC16) was the donor and B. anthracis was the recipient. It seemed as if either pBT1 or pBT2, but not both, was transferred and pBT2 was transferred (or stabilized in transcipients) much less frequently than pBT1. However, once B. anthracis acquired pBT2 it was transferred at a high frequency and the majority of transcipients were Cry^+.

Transcipients that carry either pBT1 or pBT2 are active as donors suggesting that both may be "fertility" plasmids capable of promoting the mating process. However, we have not done enough tests to rule out the possibility that one or more of the lower molecular weight plasmids that migrate faster than chromosomal DNA in agarose gels may be "fertility" plasmids. We hope to determine this in future studies.

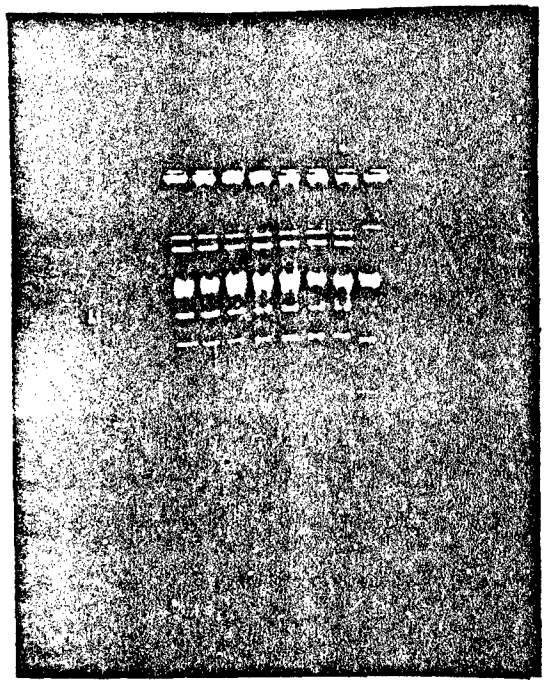


Figure 2. Agarose gel electrophoresis of plasmid extracts

Lane 1: B. thuringiensis 4042A wild type Cry^+

Lane 2: B. thuringiensis 4042A M8 Ade^- Cry^+

Lane 3: B. thuringiensis 4042A M8-13 Ade^- Spo^- Cry^-

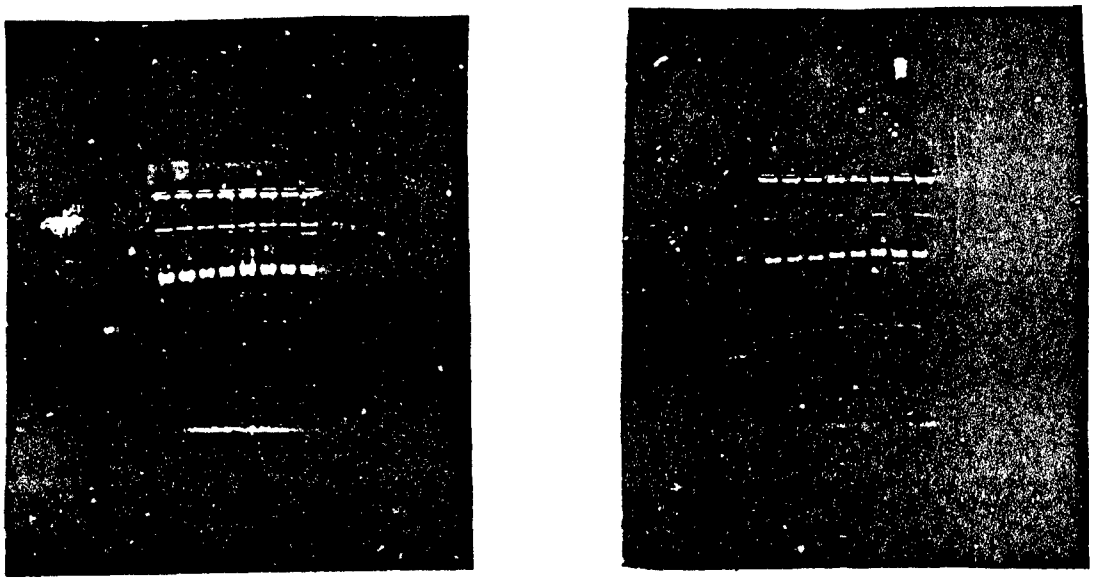
Lane 4: B. thuringiensis 4042A M8-13 td1(pBC16)

Lane 5: B. thuringiensis 4042A M8-13 td2(pBC16)

Lane 6: B. thuringiensis 4042A M8 td1(pBC16)

Lane 7: B. thuringiensis 4042A M8 td2(pBC16)

Lane 8: Cry^+ transcipient, tr 202-1, of B. cereus 569 M20 str-2 from the cross: 4042A M8 td2 x 569 M20 str-2



A B

Figure 3. Agarose gel electrophoresis of plasmid extracts from B. anthracis and B. cereus transcipients

A. B. anthracis Weybridge M44 transcipients derived from the cross: B. thuringiensis 4042A M8 td2 (pBC16) Cry$^+$ x M44 (pBA1) str-1. Lane 1, M44 tr 203-1; Lane 2, M44 tr 203-7; Lane 3, M44 tr 203-19; Lane 4, M44 tr 203-23; Lane 5, M44 tr 203-24; Lane 6, M44 tr 203-25; Lane 7, M44 tr 203-27; Lane 8, M44 tr 203-28. All were Cry$^+$ except tr 203-28 (Lane 8)

B. B. cereus 569 M20 transcipients derived from the cross: B. anthracis Weybridge A M17 tr 245-4 (pBC16)(pBA1) Cry$^+$ x B. cereus 569 M20 str-2. Wells of lanes 1 through 8 contained extracts of 569 tr 251-1 through 251-8, respectively. Tr 251-1, -2, -3, -4, -6, and -8 were Cry$^+$. Tr 251-5 and -7 were Cry$^-$.

We have no explanation for the absence of pBT2 in 4042A M8-13(pBC16) transductants. It is conceivable that the cells used for transduction were cured of the plasmid spontaneously.

Parasporal crystal formation in B. anthracis transcipients. Reproductions of photographs of crystals formed by a B. anthracis transcipient as viewed through the phase microscope are shown in Figure 4. Cry^+ B. anthracis transcipients were biologically active when compared with B. thuringiensis 4042A M8 in tests against the spruce budworm, Choristoneura fumiferana (Clemens). Cry^- B. anthracis, whether $(pBA1)^+$ or $(pBA1)^-$, had no effect in the tests. In tests for protective antigen production in hydrolyzed casein medium, shown in Table 10, Cry^+ transcipients of B. anthracis(pBA1) could not be distinguished from the parental strain.

Failure to achieve transfer of pBA1. Although B. anthracis transcipients carrying pBT2 are more effective donors of pBC16 than those carrying pBT1, it appears that the two types of transcipients are both ineffective in transferring pBA1 to either B. cereus or mutants of B. anthracis cured of pBA1. Thus far we have not been able to demonstrate reproducibly the transfer of pBA1 by means of the mating process. This is unfortunate since one of our primary goals is to develop a system for transferring pBA1. However, there are many possibilities to be explored and as we learn more about the mating process, plasmid compatibilities, and plasmid functions I am optimistic that we will find an efficient system for pBA1 transfer.

I am very encouraged by the progress we have already made in our studies of mating in B. anthracis. Our demonstration that some degree of specificity is involved in the cotransfer of plasmids between B. thuringiensis and B. anthracis, as well as between various mutants of B anthracis, suggests that further investigation of the B. thuringiensis type of mating system may be profitable.

Our survey of B. thuringiensis strains for donor activity showed that six of twelve strains tested were active in transferring pBC16 to B. anthracis. The results comparing effectiveness of the donors in transferring pBC16 to B. cereus and B. anthracis suggest that the activity of at least five of the donors differed from each other qualitatively and/or quantitatively. Certainly the three systems we have examined in some detail in B. anthracis and which originated from three different strains of B. thuringiensis were quite different from each other. Therefore, it will be important to examine the other B.

Table 10

Protective antigen production by Weybridge M44 crystal-positive transcipients

Organism tested	Protective antigen titer[1]
b. anthracis	
Weybridge M44	16
Weybridge M44 *str-1*	16
Weybridge M44 tr203-1	16
Weybridge M44 tr203-7	16
B. thuringiensis	
4042A M8 tdl(pBC16)	0

[1]Protective antigen titer is expressed as the reciprocal of the highest dilution of culture filtrate producing a visible line of precipitation in the agar diffusion assay. The antiserum was prepared in a burro by injecting Sterne spores.

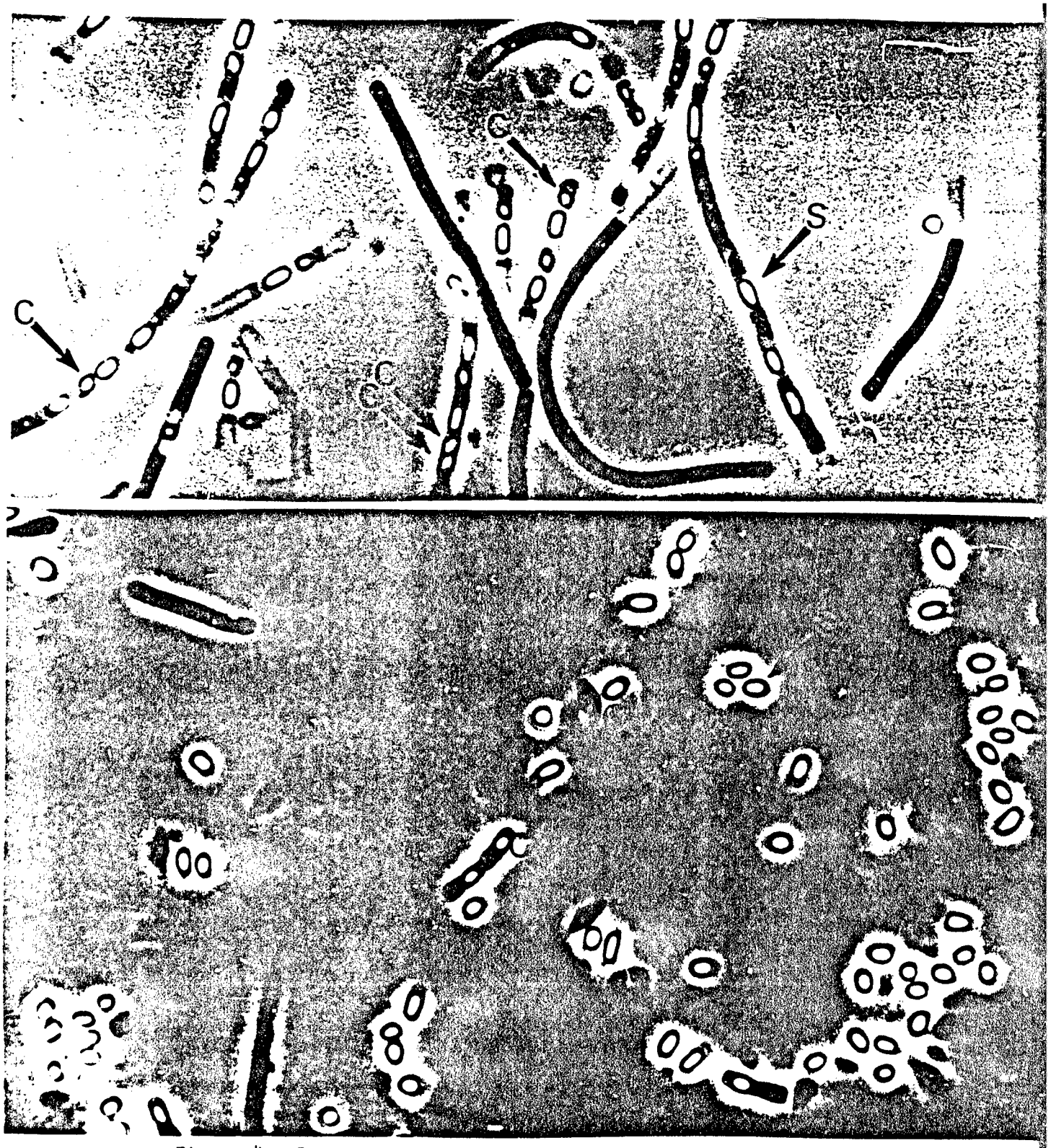


Figure 4. Parasporal crystals in B. anthracis carrying plasmids derived from B. thuringiensis

Top: Spores and crystals within cells.

Bottom: Free spores and crystals released upon lysis of a sporulating culture.

thuringiensis strains that were active in transferring pBC16 to B. anthracis. It is conceivable that one or more of them might be able to promote transfer of pBA1.

The B. anthracis donors we have tested for transfer of pBA1 have contained several plasmids. In addition to pBA1 and pBC16, they carried from two to five B. thuringiensis plasmids. It seems possible that competition might exist among plasmids during the transfer process and perhaps pBA1 is frequently excluded. Therefore, we will examine B. anthracis transcipients carefully to try to find ones that contain only pBA1, pBC16, and a single fertility plasmid. Perhaps such a donor will be able to transfer pBA1.

It might be that pBA1 and pBC16 can not cotransfer very frequently. We now have the chloramphenicol resistance plasmid, pC194, in B. anthracis and we plan to test for cotransfer of it and pBA1 by suitably constructed donors. We will move pC194 into desired B. thuringiensis donors by transduction with CP-51 and mate the transductants with B. anthracis(pBA1) Str^r. B. anthracis transcipients will be selected on agar containing streptomycin and chloramphenicol. Then representative transcipients that carry pBA1, pC194, and a fertility plasmid will be tested for their ability to transfer pBA1 to B. cereus and B. anthracis$(pBA1)^-$.

In all of our mating experiments carried out thus far we selected transcipients that acquired the drug resistance plasmid, pBC16, and then we looked among them for the presence of pBA1 by electrophoresis of DNA extracts in agarose gels. (This procedure severely limits the number of colonies that can be examined for the presence of pBA1). It is probably very likely that many transcipients are produced that do not contain pBC16, and it might be possible to identify transcipients carrying pBA1 by an immunodiffusion method. We have used such a procedure for tentatively identifying strains cured of pBA1. The procedure consists of growing colonies on hydrolyzed casein agar near wells of B. anthracis antiserum, and colonies that are $(pBA1)^+$ can be distinguished from those that are $(pBA1)^-$ on the basis of differences in lines of precipitation formed between the colonies and wells of antiserum. With frequencies of transcipients as high as 10% or more, it is feasible to use such a method to look for $(pBA1)^+$ transcipients. Mating mixtures will be plated on selective media that will allow all recipients, but not donors, to grow. Colonies chosen at random will then be tested for the presence of pBA1 by the immunodiffusion test.

Another approach we are planning is that of attempting to identify $(pBA1)^+$ B. anthracis transcipients on the basis of colonial morphology differences between $(pBA1)^+$ and cured strains. High frequencies of transfer as mentioned above would presumably make this method feasible. We will proceed as above, i.e., plate mating mixtures on selective media that will allow all recipients to grow. Colonies will then be examined for the characteristic morphology of $(pBA1)^+$ strains. The high frequencies that make this and the immunodiffusion method feasible have been obtained only with one particular mutant of B. anthracis. We do not have a cured variant of this mutant; therefore before we can use these two identification procedures we will have to isolate a cured variant. This should be easy to do by applying our curing procedure described in the Annual Report, December 1982.

Development of a mating system based on a plasmid from Streptococcus faecalis. Another possibility for transferring pBA1 lies in the use of plasmid pAM_B1, a 17 megadalton conjugative plasmid which encodes macrolide-lincosamide-streptogramin B (MLS) resistance and which was originally found in Streptococcus faecalis (6). The plasmid has been shown to have a broad host range with respect to its transferability, maintenance, and expression. It has been transferred to B. subtilis (7), and B. thuringiensis (8), and Landman et al. (7) have shown that B. subtilis could serve as a conjugal donor of pAM_B1. Lecadet, et al. (8) reported that several, but not all strains of B. thuringiensis were effective recipients of pAM_B1 from S. faecalis donors, and they presented evidence that the plasmid could mobilize a 34 megadalton cryptic plasmid present in S. faecalis. It therefore seemed that pAM_B1 might be a useful plasmid for establishing a mating system in B. anthracis. Results of our initial tests are reported here.

S. anginosis(pAM_B1) and B. subtilis(pAM_B1) were tested as donors for the transfer of pAM_B1 to B. thuringiensis, B. anthracis, and B. cereus. S. anginosis was grown for 20 hours at 37°C. in 25 ml of BHI broth in a reduced oxygen atmosphere in a candle jar. B. subtilis and the strains to be tested as recipients were grown in 25 ml of nutrient broth at 30°C. on a rotary shaker for 6 hours. The cultures were started with 0.2 ml of an overnight culture in nutrient broth. Equal volumes of donor and recipient cells were mixed together and one ml was impinged on a Millipore HA membrane. The membranes were placed on nutrient agar and incubated 48 hours at 30°C. Mating mixtures in which S. anginosis was the donor were incubated in the low oxygen atmosphere of a candle

jar; those in which B. subtilis was the donor were incubated in air. After 48 hours the cells were washed from each membrane with 0.5 ml of nutrient broth and transcipients were selected by plating 0.1-ml samples on L agar containing 25 ug of erythromycin and 1 mg of streptomycin per ml. Plates were incubated overnight at 37°C.

The results of these matings are shown in Table 11. All the strains used as recipients were sensitive to MLS antibiotics and spontaneous mutants resistant to Em were not detected. Each of the two donor strains was effective in transferring pAM_B1 to all three recipient strains. Representative transcipients derived from all three recipients were tested and found to be resistant to other MLS antibiotics, suggesting very strongly that the "transcipients" had, in fact, picked up pAM_B1 and were not resistant as a result of chromosomal mutations. Plasmid analysis showed that B. anthracis M 14 transcipients had acquired pAM_B1. Presumably additional tests will demonstrate the presence of pAM_B1 in representative transcipients from all the crosses.

We will now proceed to test whether the B. anthracis transcipients that carry pAM_B1 can serve as donors of that plasmid and other plasmids, e.g, pBA1, to B. cereus and to cells of B. anthracis cured of pBA1.

II. Influence of plasmid pBA1 on the physiology and genetics of B. anthracis

As reported previously (Annual Report, December 1982), a number of cured derivatives ($pBA1^-$) have been isolated from several genetically marked strains (auxotrophic mutants). In addition to the loss of ability to produce protective antigen, all cured strains we have tested are similar with respect to the following characteristics: (1) Cured cells sporulate earlier and at a higher frequency than uncured cells; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells are more sensitive than uncured cells to certain bacteriophages; (4) Cured cells do not grow as well as uncured cells in certain synthetic media; and (5) spore-negative mutants are found at a much higher frequency in cultures of cured strains than in $(pBA1)^+$ strains.

We have spent considerable effort in attempts to find the basic physiological and genetic differences that could account for the phenotypic changes listed above. It now seems possible that increased frequency of sporulation may be the primary altered characteristic among those listed and the

Table 11

Transfer of erythromycin resistance from S. anginosis and B. subtilis to B. anthracis, B. cereus, and B. thuringiensis

Donor	Recipient	Em<r> transcipients per ml
S. anginosis(pAM_B1	B. anthracis Weybridge M44 str-1	104
" "	B. anthracis Weybridge A M14 str-1	84
" "	B. cereus 569 M20 str-2	>500
" "	B. thuringiensis BTI str-1	>500
" "	B. thuringiensis HD-1 str-1	36
B. subtilis BQ838(pAM_B1)	B. anthracis Weybridge A M14 str-1	400
" "	B. cereus 569 M20 str-2	18
" "	B. thuringiensis BTI str-1	30

others may be direct or indirect consequences of this change.

Altered colonial morphology of cured strains is undoubtedly a direct consequence of increased sporulation. The difference in sporulation between cured and uncured strains is much more pronounced at 37°C. than at 30°C., and the change in colonial morphology is similarly much more evident with colonies grown at 37°C. than with those grown at 30°C. We and others have correlated changes in colonial morphology with altered sporulation characteristics in mutants of various other Bacillus species including B. subtilis, B. cereus, and B. thuringiensis.

It seems likely that the increased sensitivity of cured cells to bacteriophage may also be a reflection of changes in regulation of sporulation. Our original observation was that phage CP-51 plaqued with high efficiency on cells of the Weybridge strain that were cured of pBAl, but it did not form visible plaques on uncured cells. We were surprised to learn that CP-51 will plaque on spore-negative (Spo^-) mutants isolated from uncured strains as well as from cured strains. Another significant observation is that the addition of substances that inhibit sporulation, e.g. glycerol, to CP-51 phage assay medium allows plaque formation when uncured cells are used as the indicator.

Poorer growth of cured variants on minimal medium may also be a reflection of altered sporulation characteristics. On Minimal O medium colonies of cells cured of pBAl sporulate early and at high frequencies compared to uncured cells. It seems as if the amount of growth is decreased because the cells sporulate very early. Spo^- mutants derived from cured strains grow much better on Minimal O than their parent strains. When such mutants are transduced back to Spo^+ with phage CP-51, the transductants resemble the parental cured type with respect to poor growth on Mimimal O medium.

It seems doubtful that cells cured of pBAl would mutate to spore-negative more frequently than uncured cells. It seems more probable that the Spo^- mutants have a selective advantage over the Spo^+ parent; i.e., they can continue to grow after the Spo^+ cells are committed to sporulation. Strains cured of pBAl sporulate earlier than uncured strains and thus Spo^- mutants should have a greater selective advantage in cultures of cured cells than in cultures of uncured cells.

III. Isolation of mutants and chromosomal mapping

Isolation of mutants. For several reasons the isolation of auxotrophic mutants is more difficult with B. anthracis than with many other Bacillus species. In spite of such difficulty, we are gradually accumulating a collection of auxotrophic mutants which are useful for chromosomal mapping as well as for experiments in which genetically marked strains are essential. Our collection of auxotrophs, all of which have been isolated in my laboratory, now includes mutants with lesions in genes required for the synthesis of indole, tryptophan, valine, leucine, phenylalanine, histidine, arginine, purines (two or three cistrons represented), pyrimidines (at least two cistrons represented), nicotinic acid, biotin, and riboflavin.

Chromosomal mapping. In the Annual Report, December 1981, we reported two linkage groups in B. anthracis. These were in genes required for the synthesis of (1) nicotinic acid and phenylalanine and (2) uracil and carbamyl phosphate. We have recently added three additional linkages to the list. As shown in Table 12, the three new linkage groups are composed of genes involved in the synthesis of (1) leucine and histidine, (2) indole and tryptophan, and (3) histidine and purine.

Our transduction results have been improved considerably by our use of a temperature-sensitive mutant of CP-51. This mutant, ts45, does not grow or form plaques at 42°C., and its efficiency of plating at 37°C., compared with that at 30°C., is very low. By using this ts mutant and selecting transductants at 37°C., we obtain considerably more transductants. Presumably there is much less lysis of potential transductants by the ts mutant than by wild-type phage.

The use of nitrosoguanidine (NTG) for localized mutagenesis, as suggested by Oeschger and Berlyn (9), is also proving to be of value in our mapping studies. The idea of the method is to use NTG as a mutagen to revert a particular mutation, and then look among the revertants for new mutants. Since NTG is active at the site of replication, newly-induced mutations are apt to be linked to the mutation that was reverted. Among the results shown in Table 12 the linkage of his to pur was found as a result of the localalized mutagenesis method. The His^- mutant, M693, was reverted with NTG, and the Pur^- mutant, M715, was found among the His^+ revertants.

IV. Improved method for extracting plasmid DNA

In the Annual Report, December 1982, we reported our procedure for

Table 12

Cotransduction of linked markers in the Weybridge strain of B. anthracis by phage CP-51

Donor	Recipient	Cotransduction[1]
Weybridge A M11 Leu$^-$	Weybridge A M693 His$^-$	411/461 (89.3%)
Weybridge A M693 His$^-$	Weybridge A M11 Leu$^-$	205/214 (95.8%)
Weybridge A M18-M9 Ind$^-$	Weybridge A M714 Trp$^-$	114/141 (80.9%)
Weybridge A M693 His$^-$	Weybridge A M715 Pur$^-$	63/346 (18.2%)

[1]The fractions represent the number of transductants carrying the donor marker over the total number of transductants tested. The values in parentheses are per cent cotransduction obtained by multiplying the fraction by 100.

extracting and demonstrating plasmid pBA1. The method is a modification of the procedure published by Kado and Liu (10). Although the results with the modified method were far better than those we obtained with the original procedure, we still had problems. The modified procedure was quite good for large plasmids, i.e., those that migrated slower than chromosomal DNA, but it was poor for smaller plasmids which migrated faster than chromosomal DNA. In order to get a complete profile of all plasmids in a given strain we had to do two extractions, one with whole cells and one with protoplasts. Our latest modification, which is essentially an increase in the amount of alkali in the lysis buffer, alleviates this problem. We have had consistent results with our current method. Other investigators working with B. anthracis might find our modified method useful and for that reason details of the procedure are included here.

Reagents

E buffer

0.04 M Tris base

0.002 M $(Na)_4$EDTA

pH adjusted to 7.9 with glacial acetic acid

E buffer-sucrose

Sucrose (10%, w/v) prepared with E buffer

Lysis buffer

0.05 M Tris base containing 10% (w/v) sucrose

Dissolve 3 gm of sodium dodecyl sulfate in 100 ml of Tris base-sucrose and add 5 ml of 3.0 N NaOH

Phenol-chloroform

Freshly distilled phenol mixed with equal volume of chloroform

Pronase

2 mg/ml dissolved in 2 M Tris base previously adjusted to pH 7.0 with 6 N HCl

Procedure

A loop of spores or of growth from an agar plate is inoculated into 25 ml of BHI broth in a 250-ml Erlenmeyer flask which is incubated on a shaker (130 rev/min) at 37°C. for 14 to 16 hours.

Cells from 25 ml of culture are collected by centrifugation in a Sorvall SS-34 rotor at 10,000 rev/min for 10 min at 4°C. and suspended in 1 ml of E buffer-sucrose. One ml of the suspension is transferred to a tube containing 2 ml of lysis buffer. The tube is inverted 20 times to mix the cells with the lysis buffer and then held in a 55°C. water bath for 30 min. The tube is removed from the 55° bath and 0.5 ml of pronase is added. The tube is inverted 20 times and then held in a 37°C. water bath for 20 minutes. Phenol-chloroform (6 ml) is added and the tube is inverted 50 times. Finally, it is centrifuged in a Sorvall SS-34 rotor at 10,000 rev/min for 10 min at 4°C., and the aqueous layer is removed with a pipet.

V. Publications

The following paper and two abstracts have resulted thus far from the work accomplished on the contract.

(1) Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. Interspecies transduction of plasmids among Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. J. Bacteriol. 157: March 1984.

(2) The following are abstracts of papers presented at the annual meeting of the American Society for Microbiology in March 1983.

Robillard, N. J., T. M. Koehler, R. Murray, and C. B. Thorne. Effects of plasmid loss on the physiology of Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H 54, p. 115.

Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. CP-51-mediated interspecies transduction of plasmid pBC16 among Bacillus cereus, Bacillus thuringiensis, and Bacillus anthracis. Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H 76, p. 118.

The following paper, which is closely related to our work on B. anthracis, has been accepted for publication:

Barsomian, G. D., N. J. Robillard, and C. B. Thorne. Chromosomal mapping in Bacillus thuringiensis by transduction. J. Bacteriol. 157: March 1984.

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